# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

| | | |
|---|---|---|
| Applicant's or agent's file reference<br>13993-2 | **FOR FURTHER ACTION** | See Form PCT/IPEA/416 |
| International application No.<br>PCT/CA2004/001448 | International filing date *(day/month/year)*<br>30 July 2004 (30-07-2004) | Priority date *(day/month/year)*<br>14 August 2003 (14-08-2003) |

International Patent Classification (IPC) or national classification and IPC
IPC(7): C07K 1/22, C07K 1/14, G01N 33/68, G01N 33/53

Applicant
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1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. [X] *(sent to the applicant and to the International Bureau)* a total of 13 sheets, as follows:
      [X] sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      [X] sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. 1 and the Supplemental Box.
   b. [ ] *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).
4. This report contains indications relating to the following items:
   - [X] Box No. I Basis of the report
   - [ ] Box No. II Priority
   - [X] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - [ ] Box No. IV Lack of unity of invention
   - [X] Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - [ ] Box No. VI Certain documents cited
   - [X] Box No. VII Certain defects in the international application
   - [X] Box No. VIII Certain observations on the international application

| | |
|---|---|
| Date of submission of the demand<br>01 June 2005 (01-06-2005) | Date of completion of this report<br>28 December 2005 (28-12-2005) |
| Name and mailing address of the IPEA/CA<br>Canadian Intellectual Property Office<br>Place du Portage I, C114 - 1st Floor, Box PCT<br>50 Victoria Street<br>Gatineau, Quebec K1A 0C9<br>Facsimile No.: 001(819)953-2476 | Authorized officer<br>John Buchko (819) 953-5926 |

**INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY**

International application No.
PCT/CA2004/001448

**Box No. I Basis of the report**

1. With regard to the **language**, this report is based on:

[X] the international application in the language in which it was filed

[ ] a translation of the international application into , which is the language of a translation furnished for the purposes of:

[ ] international search (Rules 12.3(a) and 23.1(b))

[ ] publication of the international application (Rule 12.4(a))

[ ] international preliminary examination (Rules 55.2(a) and/or 55.3(a))

2. With regard to the **elements** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report):*

[ ] the international application as originally filed/furnished

[X] the description:

| | | | |
|---|---|---|---|
| [X] pages | 3-63 and 66-90 | | as originally filed/furnished |
| [X] pages* | 64-65a | received by this Authority on | 1 June 2005 (01-06-2005) |
| [X] pages* | 1-2 | received by this Authority on | 12 December 2005 (12-12-2005) |

[X] the claims:

| | | | |
|---|---|---|---|
| [ ] pages | | | as originally filed/furnished |
| [ ] pages* | | as amended (together with any statement) under Article 19 | |
| [X] pages* | 91-98 | received by this Authority on | 1 June 2005 (01-06-2005) |
| [ ] pages* | | received by this Authority on | |

[X] the drawings:

| | | | |
|---|---|---|---|
| [X] pages | 1-22 | | as originally filed/furnished |
| [ ] pages* | | received by this Authority on | |
| [ ] pages* | | received by this Authority on | |

[ ] a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing.

3. [X] The amendments have resulted in the cancellation of:

[X] the description, pages 1, 2, 64 and 65

[X] the claims, Nos. 1-53

[ ] the drawings, sheets/figs

[ ] the sequence listing *(specify):*

[ ] any table(s) related to sequence listing *(specify):*

4. [X] This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

[X] the description, pages 64-65

[ ] the claims, Nos.

[ ] the drawings, sheets/figs

[ ] the sequence listing *(specify):*

[ ] any table(s) related to sequence listing *(specify):*

* *If item 4 applies, some or all of those sheets may be marked "superseded."*

**INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY**

International application No.
PCT/CA2004/001448

**Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

The question whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

[ ] the entire international application

[X] claims Nos. 43, 44 and 50-52

because:

[ ] the said international application, or the said claims Nos.
relate to the following subject matter which does not require an international preliminary examination *(specify):*

[X] the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 43, 44 and 50-52
are so unclear that no meaningful opinion could be formed *(specify):*

Claims 43 and 44 do not clearly set out all the necessary steps in the methods such that a desired result can be achieved when applying the methods, as required under Article 6 PCT. Substituting amino acids which change the charge of a native polypeptide is a common method applied when studying the structure and/or function of a protein, wherein said protein may be associated with a disease. Therefore, the methods in claims 43 and 44 are so unclear and broad in scope that a meaningful written opinion with regard to novelty, inventive step and industrial applicability can not be established. Further, the reagent kits in claims 50-52 do not clearly define all the technical features of the claimed invention necessary to clearly distinguish the kits from those in the prior art. Claim 50 only defines a kit comprising a washing buffer for separating the first ligand from the second ligand, wherein the buffers are capable of maintaining the electrostatic forces between the first and second ligand. Claims 51-52 further define the kit in claim 50, however, the components of the kits in the claims appear to consist of well known components common to protein or ligand purification kits. Therefore, claims 50-52 are so broad in scope that they render the claims so unclear that a meaningful written opinion with regard to novelty, inventive step and industrial applicability can not be established.

[ ] the claims, or said claims Nos. are so inadequately supported
by the description that no meaningful opinion could be formed *(specify):*

[X] no international search report has been established for said claims Nos. 43, 44 and 50-52

[ ] a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

[ ] furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

[ ] furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

[ ] pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13*ter*.1(a) or (b) and 13*ter*.2.

[ ] a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions, and such tables were not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

[ ] the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

[ ] See Supplemental Box for further details.

**Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Claims | 1-42, and 45-49 | YES |
| | Claims | | NO |
| Inventive step (IS) | Claims | 1-42, and 45-49 | YES |
| | Claims | | NO |
| Industrial applicability (IA) | Claims | 1-42, and 45-49 | YES |
| | Claims | | NO |

2. Citations and explanations (Rule 70.7)

D1: BAUER and KUSTER. "Affinity purification-mass spectrometry: Powerful tools for the characterization of protein complexes." Eur. J. Biochem. vol. 270, pages 570-578, February, 2003.
D2: RIGAUT et al. "A generic protein purification method for protein complex characterisation and proteome exploration." Nature Biotech. vol. 17, pages 1030-1032, October, 1999.
D3: FORMOSA et al. "Using protein affinity chromatography to probe structure of protein machines." Methods Enzymol. vol. 208, pages 24-45, 1991.
D4: WO 02056025 (AWREY and GREENBLATT), 18 July 2002.

Novelty:

D1 discloses a method of purifying and identifying component parts of protein complex comprising expressing a ligand fused to a tandem affinity purification (TAP) tag in a cell. Complexes containing the fusion ligand are purified sequentially via two independent affinity binding steps on IgG and calmodulin containing resins. The component parts of the complex are then identified using mass spectrometry. The method only elutes protein-protein complexes from the affinity matrix. D1 further discloses other generally known methods of affinity purification (e.g. immobilized antibodies, epitope tagging, GST pull-down and biotinylated RNA tagging).

D2 discloses a method of purifying and identifying component parts of protein complexes comprising expressing a ligand fused to a TAP tag in a cell. Complexes containing the fusion ligand are purified sequentially via two independent affinity binding steps on IgG and calmodulin containing resins. The component parts of the complex are then identified using mass spectrometry.

D3 discloses general methods of purifying and identifying ligands using affinity chromatography comprising immobilizing a ligand or protein to a solid support, coupling a test sample or cell extract to the ligand-immobilized support, eluting bound components (i.e. protein or ligand molecules in the test sample or cell extract) by decreasing the electrostatic forces between the components and immobilized ligand (see page 34, last paragraph), and characterizing the eluted components.

D4 discloses methods for the purification and identification of interacting protein in a protein-protein complex comprising immobilizing a protein ligand to a support matrix, subjecting a cell extract or test sample to affinity chromatography (two or more columns), eluting interacting proteins, which have bound to the immobilized ligand, by decreasing the electrostatic forces between the interacting proteins and immobilized ligand (see page 29, lines 3-6), and analysing the interacting proteins by mass spectrometry.

D1 and D2 each disclose a method wherein a complex comprising a first ligand and second ligand, fused to at least two different affinity tags, is bound to a matrix support. However, D1 and D2 do not disclose the elution of only one of the ligands from the affinity matrix.

(continued in Supplemental Box)

**Box No. VII** **Certain defects in the international application**

The following defects in the form or contents of the international application have been noted:

The term "pf"in claim 37 should probably read "of."

**Box No. VIII Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Contrary to the requirements of Article 6 PCT, there is no substantive support given in the current description for claims 45 and 46 for a method for identifying the putative cause for a disease or any specific disease. The description only provides substantive support for the general purification and identification of ligands in a protein complex and does not identify any specific protein-protein associations which correspond with a specific disease. The use of the claimed method for identifying complexes associated with a disease is speculative, based on discoveries in the current application and the prior art.

Contrary to the requirements of Article 6 PCT, Claims 43 and 44 do not clearly set out all the necessary steps in the method such that a desired result (identifying protein-protein association as a putative cause for a disease) can be achieved when applying the methods.

Contrary to the requirements of Rule 6.3(a) PCT, the reagent kits in claims 50-52 do not clearly define the claimed invention in terms of their technical features. Claim 50 only defines a kit comprising a washing buffer agent for separating the first ligand from the second ligand wherein the buffer are capable of maintaining the electrostatic forces between the first and second ligand. The kits in claims 50-52 comprise components known in the art which are common to protein or ligand purification kits, therefore, they are not distinguishable from the prior art (i.e. lacking technical features). Further, the kit components in claims 50-52 only appear to embody functional features (i.e. "capable of maintaining the electrostatic forces between the first and the second ligand"and "the chemical agent is capable of decreasing the electrostatic forces between the first and the second ligand") and do not clearly define specific component in terms of their specific chemical compositions. Therefore, claims 50-52 do not comply with Article 6 PCT, due to the use of broad functional language which does not clearly and concisely define the claimed kits.

**Supplemental Box**

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of: Box No. V

D3 and D4 each disclose a method wherein a cell extract or test sample are subjected to affinity chromatography. However, only a single ligand is immobilized to a support matrix before treating the matrix with an extract or sample, followed by elution of bound ligands. Therefore, D3 and D4 do not teach the immobilization of second ligand to a matrix which is already bound to a first ligand.

Therefore claims 1-42, and 45-49 are considered to be novel, as required under Article 33(2) PCT, in view of D1, D2, D3 and D4.

**Inventive step:**

The current invention is based on the discovery that in vivo formed transient complexes are associated by predominately electrostatic bonds and that the disruption of the electrostatic bonds can be used for the purification of a ligand from a complex. The current invention appears to solve the problem of detecting substoichiometrically interacting proteins which make up the transient complexes. D1 and D2 each disclose a general method for immobilizing a first and second ligand complex to a support, wherein one of the ligands is fused to at least two different affinity tags. D1 and D2 each differ from the present application in that the whole complex is eventually eluted from the matrix. Therefore D1 and D2 do not teach the elution of one of the ligands by decreasing the electrostatic interactions between them. D3 and D4 each disclose the elution of only a single ligand which is complexed to a second ligand by decreasing the electrostatic forces between the first and second ligand. D3 and D4 each differ from the present application in that only a single ligand, which is not bound to a second ligand, is immobilized to a support matrix (which already has a ligand bound to it), instead of immobilization of a ligand-ligand complex to the matrix. Therefore, D3 and D4 teach away from immobilizing a pre-formed complex, comprising a first and second ligand, to a matrix support. In addition D1, D2, D3 and D4 do not appear to disclose the separation of in vivo formed transient complexes by increasing the ionic strength (i.e. decreasing the the electrostatic forces between the ligands which form the complex). Each of D1, D2, D3 and D4, or viewed together, teach away from the invention in the current application. Therefore, claims 1-42, and 45-49 contain an inventive step, as required under Article 33(3) PCT, in view of D1 or D2 and D3 or D4.

**Industrial applicability:**

Claims 1-42, and 45-49 meet the requirements under Article 33(4) PCT for industrial applicability.

**Supplemental Box**

**In case the space in any of the preceding boxes is not sufficient.**

Continuation of: Box 1, Item 4

The amendments, received by this authority on 1 June 2005, included on page 64 line 18- page 65, line 14 contains subject matter which goes beyond the disclosure of the international application as filed. Nowhere in the originally filed application were the following proteins disclosed as being identified using the claimed invention: Pap1p, Ume6p, Mss4p, Nrg1p, Eco1p, Pwp1p, Ppn1p, Stm1(YLR150W), Stb6b, Mcm2p, Mcm3p, Mcm6p, Rif2p, Top2p, Set7p, Hir3p, Pho85p, Ylr114cp, Hex3p0, Sin3p, Sgd1p, Tom1p, Vik1p, Ctk2p, Ydr466wp, Ssn3p, Hpr1p, Ydr527wp, Spt3p, Ppm1p, Gal4p, Adr1p, Ime2p, Rsc4p, Shu2p, Rox3p, Rna14p, Dcp1p, Hfi1p, Dem1p, Sip5p, Hir3p, Ada2p, Rlr1p, Prp42p, Toa2p(TFIIA), Pat1p, Taf7p, Yil137cp, Hcm1p, Ctf13p, Cdc15p, Swr1, Smf2p, Cup2p, Lrp1p, Ybr022wp, Afg1p, Gea2p, Yor004wp, Ycr030cp, Ycr090cp, Ymr086wp, Ybr255wp, Yjr072cp, Ynl155wp, Rad50p, Ctf4p, Ufd4p, Rom2p, Pfs2p, Ino4p, Ynr029cp, Ymr098cp, Skt5p, Yjr154wp, Pnp1p, Ydr051cp, Mkt1p, Yar069cp, Yll040cp, Gif1p, Ypr127wp, Sco2p, Ydr179wp, Psk2k, Ynl313cp, Rok1p, Yol160wp, Cdc47p, Cdc45p, Kog1p, Cdc23p, Cdc54p, Nog2p and Dus3p.

This report has been established as if the above indicated subject matter has not been included in the application, under Rule 70.2(c) PCT, since it is considered to embody new subject matter not disclosed in the originally filed application,

**TITLE:** Method for detecting transient protein interactions and drug discovery

**FIELD OF THE INVENTION**

The invention relates to methods for separating substoichiometrically interacting proteins or other ligands from immobilized ligand of interest in an affinity matrix system. In addition, the invention can be used as a method for drug discovery.

**BACKGROUND OF THE INVENTION**

An important scientific pursuit in the post-genomic era is to determine the components of multiprotein complexes and to identify the protein-protein interactions in a proteome. The proteome consists of stable protein complexes and single (free) proteins but virtually every cellular process is mediated by and/or consists in transient protein interactions, e.g. protein-protein interactions, DNA-protein interactions or RNA-protein interactions. The detection and analysis of the transient interactions is a major challenge in biology and proteomics.

One method for protein purification and detection of protein interactions is creating fusion proteins by using recombinant DNA techniques. For example, the fusion protein can contain a protein of interest and an affinity tag capable of binding selectively to an affinity matrix. After expressing the fusion protein in exogenous or endogenous organism, it is immobilized on an affinity matrix and the unbound substances are removed. Subsequently, the fusion protein is released from the affinity matrix. The method is used in order to purify the affinity tagged protein or, more recently, the affinity tagged protein and the proteins that associate with it. The advent of sensitive mass spectrometric methods for protein identification and improved affinity purification methods made possible the direct identification of protein complexes on a proteome-wide scale (Ho Y. et al Nature 2002, Gavin, A.C. et al. Nature 2002). A review by R. Aebersold and M. Mann, Nature, March 2003, Vol. 422, "Mass spectrometry-based proteomics" describes in more detail the state of the field.

More than 300 stable complexes have been identified in the model organism *Saccharomyces cerevisiae* and extensive proteome maps have been built. However, it became clear that the number of transient protein-protein interactions detected by affinity purification coupled with mass spectrometry is smaller (often by an order of magnitude) compared to the number of transient protein-protein interactions detected by genetic based methods and library based methods. This is due to the fact that when the fusion protein forms transient complexes with other proteins, the latter are isolated in substoichiometric amounts and most often are not detected.

When the fusion protein and the interacting proteins are isolated via an affinity tag, the bulk of the fusion protein is immobilized on the affinity matrix, whereas only that fraction of the interacting proteins which has been bound to the fusion protein *in vivo* is immobilized. When the fusion protein is released from the affinity matrix, the ratio between the proteins in the eluate remains the same and, as a result, the highly abundant fusion protein suppresses the identification and analysis of the substoichiometrically interacting proteins. The detection of transient protein-protein interactions (e.g. interactions between stable protein complexes or between two single proteins or between a stable protein complex and a single protein) is still very difficult and in most cases impossible. Examples of proteins involved in transient protein-protein interactions include protein substrates, modifying enzymes such as protein kinases, signal transducers, DNA polymerase, replication factors, RNA polymerase, and transcription factors.

The following example with RNA polymerase II and the transcription factors illustrates the difficulty of detecting transient protein-protein interactions and the general problem of detecting any transient protein interaction by using any affinity tagged protein from any organism.

Eukaryotic RNA polymerase II is a permanent complex of twelve subunits and it forms transient complexes with different transcription factors. When a SpA-tagged subunit of RNA Polymerase II core complex from *Saccharomyces cerevisiae* is expressed under physiological conditions, it associates permanently with other subunits of the core complex and, as a part

helicase-like protein, Prp18 - helicase-like protein, Cdc6 - GTPase/ATPase; cell cycle control, Ygr054 - part of Swi/Snf and Npl3 complexes, Sdc25 - GDP/GTP exchange factor for Ras, Glo3 - GTPase activator,

(d) other proteins: Sap4 - associates with the SIT4 phosphatase, Sap185 - SIT4 associated protein, Ptc2 - protein phosphatase type 2C, Ubp8 - putative deubiquitinating enzyme, Kin4 - nuclear protein kinase, Hbt1 - Hub1 target, Yhr121w - Stb5 associated, Sup35 - interacts with Mip6 and Nab3, Sip1 - Snf1 protein kinase substrate, Rtt103 – binds to CTD, Mks1, Ydl145 (Cop1), Mpd2 - protein disulfide isomerase. More proteins are indicated in the Examples.

The nucleotide sequences of the genes and amino acid sequences of the proteins can be found by submitting the ORF name or the name of the protein at: Saccharomyces Genome Database at yeastgenome.org/ or, at National Center for Biotechnology Information web site at ncbi.nlm.nih.gov:80/mapview/map_search.cgi?taxid=4932.

In total, by practicing the method of the invention with different affinity tagged subunits of RNA Polymerase II core enzyme from Saccharomyces cerevisiae we have isolated and identified the following proteins:

Tfg1p, Tfg2p, Tfg3p, Fcp1p, TFIIS, TFIIB, Tfa2p(TFIIE), Tfb2p(TFIIH), Spt5p, Spt6p, Iws1p, Cet1p(β), Ceg1p(α), Rgr1p, Med4p, Med1p, Srb4p, Srb8p, Med7p, Rtf1p, Paf1p, Ctr9p, Leo1p, Cdc73p, Spt16, Pob3p, Ess1p, Rgt1p, Iwr1p, Prp8p, Taf5p, Taf9p, Taf12p, Rad3p, Fip1p, Pap1p, Cft1p, Hrp1p, Clp1p, Prp43p, Syf1p, Smb1p, Prp18p, Prp22p, Puf2p, Ume6p, Mss4p, Nrg1p, Eco1p, Pwp1p, Rgm1p, Ppn1p, Nip1p, Nhp2p, Npl3p, Rtt103p, Rai1p, Ptc1p, Ptc2p, Ptc3p, Set2p, Set3p, Stm1(YLR150W), Mbf1p, Mks1p, Stb5p, Stb6p, Mcm2p, Mcm3p, Mcm6p, Nam7p, Rap1p, Rif1p, Rif2p, New1p, Cdc6p, Cyr1p, Sdc25p, Rad1p, Rad23p, Ddr48p, Ddc1p, Din7p, Tof1p, Top2p, Ubp8p, Tho1p, Mpd2p, Sup35p, Hbt1p, Sip1p, Sap4p, Sap185p, Set7p, Hir3p, Pho85p, Ylr114cp, Hex3p, Sin3p, Sgd1p, Tom1p, Cop1p, Glo3p, Vik1p, Ctk2p, Stt4p, Ygr054wp, Yhr121wp, Ybr262cp, Ylr419wp, Yor379cp,

Yol045p, Ygr086cp, Ybr174cp, Yol022cp, Ydr466wp, Sub1p, Fun11p, Cdc39p, Ssn3p, Cdc48p, Kin1p, Kin4p, Clu1p, Hpr1p, Pcf1p, Mrt4p, Lte1p, Pbp1p, Has1p, Hpr5p, Prp2p, Prp6p, Prp16p, Ydr527wp, Spt3p, Ppm1p, Gal4p, Adr1p, Ime2p, Rsc4p, Shu2p, Rox3p, Rna14p, Dcp1p, Hfi1p, Dem1p, Sip5p, Hir3p, Rpd3p, Ada2p, Rlr1p, Drs1p, Prp42p, Toa2p(TFIIA), Top1p, Pat1p, Taf7p, Yil137cp, Hcm1p, Ctf13p, Cdc15p, Swr1p, Smf2p, Cup2p, Lrp1p, Ybr022wp, Afg1p, Gea2p, Sec21p, Yor004wp, Ycr030cp, Ycr090cp, Ymr086wp, Ybr255wp, Yjr072cp, Ynl155wp, Rad50p, Ctf4p, Ufd4p, Rom2p, Pfs2p, Ino4p, Ynr029cp, Ymr098cp.

We have also identified with somewhat lower confidence 25 other proteins: Skt5p, Yjr154wp, Pnp1p, Ydr051cp, Mkt1p, Yar069cp, Bim1p, Yll040cp, Gif1p, Ypr127wp, Sco2p, Ydr179wp, Psk2p, Ynl313cp, Rok1p, Yol160wp, Cdc47p, Ydr065wp, Cdc45p, Kog1p, Cdc23p, Gcd14p, Cdc54p, Nog2p, Dus3p.

Note that by using only one method, i.e. the method of the invention, it is possible to identify directly nearly all proteins that are known to interact with RNA Polymerase II and that were identified during the last 20 years by using various methods (including genetic based and library based methods).

Note that among the detected proteins are two well-known enzymes that modify covalently the carboxyl-terminal domain (CTD) of RNA Polymerase II: (a) Fcp1 – a TFIIF interacting phosphatase that recycles RNA Polymerase II, (b) Ess1 – a prolyl isomerase of the CTD. Since post-translational modifications play a major role in modulating the protein function, the identification of the modifying enzyme for a protein of interest is an important application of the invention. In addition, the invention can be used to identify the substrate for an enzyme of interest.

The presence of many (more than 30) well-established transcription factors in the high salt eluates obtained from immobilized RNA Polymerase II is enough for validation of the invention, i.e. the proteins isolated by the method of the invention are true interacting proteins. One way to validate interacting proteins is to perform the method of the invention with a fusion protein containing the putative interacting protein. The presence of the first

method of the invention are true interacting proteins. One way to validate interacting proteins is to perform the method of the invention with a fusion protein containing the putative interacting protein. The presence of the first protein of interest among the interacting proteins increases the confidence that the protein-protein interaction is physiologically relevant. For example, by performing the method of the invention with tagged subunits of RNA Polymerase II, the three subunits of the transcription factor TFIIF (Tfg1, Tfg2 and Tfg3) are detected in the high salt eluate, and, on the other hand, by performing the method of the invention with tagged Tfg1, the subunits of the RNA Polymerase II core enzyme are detected (Figures 7 and 8).

Alternatively, the validation can be performed by other methods for detection of protein interactions as described by Phizicky E. and Fields S. in Microbiological Reviews, Mar. 1995, Vol. 95, No. 1, or as described by Phizicky E, Bastiaens PI, Zhu H, Snyder M, Fields S., Nature 2003. For example, purification via an affinity tag fused to the putative interactor can be performed. Rtt103 is among the interacting proteins detected by the method of the invention and, in order to validate it as an interactor, a tandem affinity purification was performed with a strain expressing TAP-tagged Rtt103. As shown in Figure 22, two subunits of the RNA Polymerase II core complex (Rpb1 and Rpb2) co-purified with Rtt103. Legend: 470 - Rpb1, 471 - Rpb2, 472 – Rat1, 473 - Rtt103, 474 - Rai1.

**Example 5: Drug Screening and Drug Discovery**

The invention can be used for monitoring the effect of a pre-drug on a protein-protein interaction that is associated with a disease. The protein can be fused to a SpA-tag but if the cellular lysate or other biological fluid contains a high level of immunoglobulins or other proteins that bind to SpA-tag or IgG, a different affinity tag should be used (e.g. MBP or SBP or GST). In this example, the protein does not contain a pathogenic mutation. It is best if a complete proteomic map has been built around the protein of interest before the experiment, so that the presence of interacting proteins in the eluate can be determined by immunoassays or other sensitive methods.

**WE CLAIM:**

1. A method of separating a first ligand from a second ligand in an affinity matrix system, wherein the first ligand associates *in vivo* with the second ligand and wherein the first ligand and the second ligand associate with each other by electrostatic forces, comprising the steps:
   (a) Obtaining a sample containing biological complexes that include the first ligand and the second ligand;
   (b) Immobilizing the second ligand on an affinity matrix;
   (c) Removing unbound substances from the affinity matrix;
   (d) Separating the first ligand from the immobilized second ligand, which remains bound to the affinity matrix during the separation, by decreasing the electrostatic forces between the first ligand and the second ligand; and
   (e) Optionally, analyzing the separated first ligand.
2. A method of separating a first ligand from a second ligand in an affinity matrix system, wherein the first ligand associates *in vivo* with the second ligand, wherein the first ligand and the second ligand associate with each other by electrostatic forces, and wherein the second ligand is a recombinant fusion protein containing at least one affinity tag, comprising the steps:
   (a) Introducing into a cell or organism a recombinant nucleic acid molecule encoding a fusion protein comprising the second ligand fused to at least one affinity tag that can selectively bind to an affinity matrix;
   (b) Expressing the fusion protein;
   (c) Obtaining a sample containing biological complexes that include the first ligand and the fusion protein;
   (d) Immobilizing the fusion protein on the affinity matrix via the affinity tag;
   (e) Removing unbound substances from the affinity matrix;

(f) Separating the first ligand from the immobilized fusion protein, which remains bound to the affinity matrix during the separation, by decreasing the electrostatic forces between the first ligand and the fusion protein; and

(g) Optionally, analyzing the separated first ligand.

3. A method of separating a first ligand from a second ligand in an affinity matrix system, wherein the first ligand associates *in vivo* with the second ligand, wherein the first ligand and the second ligand associate with each other by electrostatic forces, and wherein the second ligand is a recombinant fusion protein containing at least two different affinity tags, comprising the steps:

(a) Introducing into a cell or organism a recombinant nucleic acid molecule encoding a fusion protein comprising the second ligand fused to at least two different affinity tags that can selectively bind to different affinity matrixes;

(b) Expressing the fusion protein;

(c) Obtaining a sample containing biological complexes that include the first ligand and the fusion protein;

(d) Immobilizing the fusion protein on a first affinity matrix via a first affinity tag;

(e) Removing unbound substances from the first affinity matrix;

(f) Separating the fusion protein from the first affinity matrix;

(g) Immobilizing the fusion protein on a second affinity matrix via a second affinity tag, which is different than the first affinity tag;

(h) Removing unbound substances from the second affinity matrix;

(i) Separating the first ligand from the immobilized fusion protein, which remains bound to the second affinity matrix during the separation, by decreasing the electrostatic forces between the first ligand and the fusion protein; and

(j) Optionally, analyzing the separated first ligand.

4. A method of separating a first ligand from a second ligand in an affinity matrix system, wherein the first ligand associates *in vivo* with the second ligand, wherein the first ligand and the second ligand associate with each other by electrostatic forces, and wherein the second ligand is a protein complex containing two or more subunits of which are fused to different affinity tags, comprising the steps:
   (a) Introducing into a cell or organism recombinant nucleic acids molecules encoding fusion proteins comprising the two or more subunits of which are fused to different affinity tags that can selectively bind to different affinity matrixes;
   (b) Expressing the fusion proteins;
   (c) Obtaining a sample containing biological complexes that include the first ligand and the fusion proteins;
   (d) Immobilizing the fusion proteins on a first affinity matrix via a first affinity tag;
   (e) Removing unbound substances from the first affinity matrix;
   (f) Separating the fusion proteins from the first affinity matrix;
   (g) Immobilizing the fusion proteins on a second affinity matrix via second affinity tag, which is different than the first affinity tag;
   (h) Removing unbound substances from the second affinity matrix;
   (i) Separating the first ligand from the immobilized fusion proteins, which remain bound to the second affinity matrix during the separation, by decreasing the electrostatic forces between the first ligand and the fusion proteins; and
   (j) Optionally, analyzing the separated first ligand.
5. The method according to any one of claims 1, 2, 3 or 4, wherein the second ligand is immobilized selectively on the affinity matrix coated with antibody that binds to the second ligand.
6. The method according to claim 2, wherein at least one affinity tag can bind selectively to the Fc domains of immunoglobulin.

7. The method according to claim 6, wherein the at least one affinity tag contains one or more IgG binding regions of Staphylococcus aureus Protein A or Streptococcal protein G.
8. The method according to claim 2, wherein the at least one affinity tag can be separated selectively from the affinity matrix by treatment with a chemical agent.
9. The method according to claim 8, wherein the at least one affinity tag is selected from the group consisting of GST-tag, SBP-tag, calmodulin binding peptide and maltose binding peptide.
10. The method according to any one of claims 3 or 4, wherein the second ligand is separated from the first affinity matrix by enzymatic cleavage.
11. The method according to claim 10, wherein the enzymatic cleavage is cleavage by TEV protease.
12. The method according to any one of claims 3 or 4, wherein the immobilization in step (d) is performed by binding to a solid support coated with a specific antibody and the removal in step (e) is performed by addition of the same antibody.
13. The method according to any one of claims 3 or 4, wherein the immobilization in step (g) is performed by binding to a solid support coated with a specific antibody.
14. The method according to any one of claims 3 or 4, wherein the first affinity tag can bind selectively to the Fc domains of immunoglobulin.
15. The method according to claim 14, wherein the first tag contains one or more IgG binding regions of Staphylococcus aureus Protein A (SpA-tag) or Streptococcal protein G (SpG-tag).
16. The method according to any one of claims 3 or 4, wherein the first affinity tag can be separated selectively from the first affinity matrix by treatment with a chemical agent.
17. The method according to claim 16, wherein the first affinity tag is from the group consisting of GST-tag, SBP-tag, calmodulin binding peptide and maltose binding peptide.

18. The method according to any one of claims 3 or 4, wherein the second affinity tag can be separated from the first affinity matrix by treatment with a chemical agent.
19. The method according to claim 18, wherein, the second affinity tag is from the group consisting of GST-tag, SBP-tag, calmodulin binding peptide and maltose binding peptide.
20. The method according to any one of claims 3 or 4, wherein the second affinity tag can bind selectively to the Fc domains of immunoglobulin.
21. The method according to claim 20, wherein the second tag contains one or more IgG binding regions of Staphylococcus aureus Protein A (SpA-tag) or Streptococcal protein G (SpG-tag).
22. The method according to any one of claims 1 to 21, wherein the first and second ligand are separated by decreasing the electrostatic forces between the first and second ligand by increasing the ionic strength of the system.
23. The method according to claim 22, wherein the ionic strength of the system is increased with a chemical agent.
24. The method according to claim 23, wherein the chemical agent is KCl.
25. The method according to claim 23, wherein the change of the concentration of the chemical agent is less than 30 mM.
26. The method according to claim 23, wherein the change of the concentration of the chemical agent is between 30 mM and 300 mM.
27. The method according to claim 23, wherein the change of the concentration of the chemical agent is between 300 mM and 700 mM.
28. The method according to claim 23, wherein the change of the concentration of the chemical agent is between 700 mM and 2 M.
29. The method according to any one of claims 1 to 21, wherein the first and second ligand are separated by decreasing the electrostatic forces between the first and second ligand by changing the pH of the system.

30. The method according to any one of claims 1 to 21, wherein the first ligand is separated from the second ligand by enzymatic treatment that modifies the second ligand.

31. The method according to any one of claims 1 to 21, wherein the first ligand is separated from the second ligand by enzymatic treatment that modifies the first ligand.

32. The method according any one of claims 1 to 31, wherein after the separation of the second ligand from the first ligand, the immobilized second ligand is mixed with a cellular lysate from a different or same organism, and after removal of the unbound substances, the first ligand is separated from the second ligand.

33. The method according to claim 32, wherein an affinity tagged second ligand is covalently cross-linked to the affinity matrix after the separation of the second ligand from the first ligand and before the immobilized second ligand is mixed with a cellular lysate from a different or same organism.

34. The method according to any one of claims 1 to 31, wherein a chemical or biomolecule is identified as a drug or pre-drug by its capability to affect selectively the separation of the first ligand from the second ligand when it is added to or removed from the cellular lysate.

35. The method according to claim 34, wherein the second ligand is a protein which contains at least one mutation.

36. The method according to claim 34, wherein the second ligand associates directly or indirectly with a protein that contains at least one mutation.

37. The method according to claims 34, wherein the chemical or biomolecule is designed, synthesized and/or selected for testing by the capability pf the chemical or biomolecule to bind to the first ligand or second ligand.

38. The method according to any one of claims 34-37, wherein the chemical or biomolecule is designed, synthesized and/or selected for testing by one of the following features: (a) capability of the chemical or biomolecule to bind selectively to the mutated protein; (b) the chemical or biomolecule contains at least one electrostatic charge that is identical to the charge

that has been changed as a result of the mutation, and (c) after binding of the chemical or biomolecule to the mutated protein, a electrostatic charge is located at distance between 0 and 0.5 nanometers from a mutated amino acid in the mutated protein.

39. The method according to any one of the claims 1 to 38, wherein the second ligand is an enzyme.

40. The method according to any one of the claims 1 to 38, wherein the second ligand is a substrate for an enzyme.

41. The method according to any one of the claims 1 to 38, wherein the second ligand is an enzyme and a substrate for a different enzyme.

42. The method according to claim 41, wherein the second ligand is RNA polymerase or DNA polymerase.

43. A method for identifying protein-protein association as a putative cause for a disease, comprising associating the disease with a mutation that changes the electrostatic properties of a protein by replacing Lysine and/or Arginine and/or Aspartate and/or Glutamate and/or Histidine with an uncharged or oppositely charged amino acid.

44. A method for identifying protein-protein association as a putative cause for a disease, comprising associating the disease with a mutation that changes the electrostatic properties of a protein by replacing an amino acid with Lysine and/or Arginine and/or Aspartate and/or Glutamate and/or Histidine.

45. The method according to any one of claims 1 to 31, wherein protein-protein association as a putative cause for a disease is identified.

46. The method of claim 45, wherein the disease comprises Norrie disease, Alzheimer's disease, Parkinson's disease, beta3-adrenergic receptor gene mutation, achondroplasia, sickle cell anemia, thrombosis, or alpha 1-antitrypsin deficiency.

47. The method according to claims 1-4, wherein the second ligand is a glycoprotein and the affinity matrix is lectin coated beads.

48. The method according to claims 1-4, wherein the second ligand is a nucleic acid, which is part of a nucleoprotein complex and the affinity matrix, consists of immobilized nucleic acid with complementary sequence.

49. The method according to claims 1-4, wherein the second ligand is a nucleic acid, which is part of a nucleoprotein complex and is genetically engineered so that it contains a poly-Guanosine and the affinity matrix consists of immobilized poly-dCytosine.

50. A reagent kit comprising a buffer for preparation of cellular lysate and washing buffer and wherein the buffers are capable of maintaining the electrostatic forces between the first ligand and the second ligand.

51. A reagent kit according to claim 50 wherein the ionic strength of the buffers is between 0 and 400 mM.

52. A reagent kit according to any one of claims 50 and 51 additionally comprising at least one chemical agent for separating the first ligand from the second ligand and wherein the chemical agent is capable of decreasing the electrostatic forces between the first ligand and the second ligand.